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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

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Additional inventors are being named on the _____ separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)

LIVE BACTERIA FOR THE PREVENTION AND TREATMENT OF POST-WEANING DIARRHEA ASSOCIATED WITH ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC) AND FOR GROWTH PROMOTION IN PIGS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

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Date February, 2, 2004

REGISTRATION NO. 53,917 *(if appropriate)*

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LIVE BACTERIA FOR THE PREVENTION AND TREATMENT OF POST-WEANING DIARRHEA ASSOCIATED WITH ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC) AND FOR GROWTH PROMOTION IN PIGS

FIELD OF THE INVENTION

The present invention relates to an orally administered live *E. coli* strain for the prevention of the post-weaning diarrhea (PWD) in pigs and to promote growth in healthy animals, such as pigs. This strain is a non-enterotoxigenic strain expressing the F4 (or K88) attachment factor.

BACKGROUND OF THE INVENTION

ETEC strains are recognized as the main causative agent of diarrhea and mortality for newborn piglets (neonatal diarrhea) and weaned pigs (PWD) (Fairbrother, 1992). ETEC strains involved in these diseases are mainly identified as serotype O149 and generally produce the F4 attaching factor and a combination of several toxins, such as LT, STa and Stb (Fairbrother, 1992). F4 fimbriae mediate adhesion of the bacteria to specific receptors (F4 receptors) on the brush borders of porcine intestinal epithelial cells. The absence or presence of these receptors is based on genetic inheritance. The adhesion of F4 fimbriae to receptors of intestinal cells is necessary for the colonisation of the intestine by F4-positive ETEC. Pathogenesis of F4-positive ETEC associated diseases includes several steps. After introduction of the F4-positive ETEC bacteria into the pig intestines, they colonize the intestine by the adhesion of F4 fimbriae to specific receptors. Bacteria start to intensively multiply and to transfer toxins into intestinal cells. The action of the toxins will cause the diarrhea (Fairbrother, 1992). Non-enterotoxigenic F4-positive bacteria will also colonize the intestines but without causing the disease. F4 fimbriae are strongly immunogenic and induce a protective immune response in pigs (van den Broeck, 1999). During infection, mucosal anti-F4 antibodies can prevent the intestinal colonization of F4-positive ETEC.

Several vaccines exist for the prevention of neonatal diarrhea in pigs. However, there is no efficacious product available for the prevention of PWD. Neonatal diarrhea is mainly controlled by vaccination of the sow (mother) with F4 fimbriae. Several approved injectable vaccines are available for sows. Intramuscular injection of sows induces systemic anti-F4 antibody production. These antibodies are thus transferred to

the colostrum and the milk, protecting piglets during nursing. Anti-F4 antibodies will prevent the adhesion of F4 fimbriae to specific intestinal receptors, preventing the bacterial colonization. During the first 2 weeks of life, piglets have no systemic mature antibodies and are fully dependant on antibodies in the colostrum and milk for their protection against pathogenic bacteria (Bijlsma et al., 1987).

At weaning, generally at 21 to 28 days of age, piglets are deprived of antibodies in the milk and become vulnerable to ETEC. The control of PWD is still an important challenge in pig production. This disease is one of the most important causes of economic losses in pig production. Diarrhea, growth delay, recrudescence of concomitant diseases, drug cost, and mortality are responsible for these economic losses. ETEC associated mortality levels vary from 10 to 30% during the post-weaning period. Furthermore, ETEC associated with porcine PWD show high levels of antibiotic resistance (Fairbrother et al., personal communication). Analyses done on porcine ETEC isolated from 1978 to 2000 in Quebec (Canada) revealed a dramatic raise of antibiotic resistance since 1996. Some strains were resistant for all the 10 tested antimicrobial agents.

A recrudescence of PWD in pigs has been observed during the last 10 years in all pig producing countries. The exact causes for this recrudescence are still unknown but may be due to the emergence of more pathogenic and more antibiotic-resistant *E. coli* strains and/or husbandry changes (early weaning) and/or new European regulations forbidding use of antimicrobial agents as growth promoters or for prophylaxis treatment (prevention of diseases) and use of high levels of heavy metals, such as zinc oxide in the feed (www.ucsusa.org/food_and_environment/antibiotic_resistance_archive/page.cfm?pageID=259). North American pig producers use high levels of antibiotics and zinc oxide to try to control PWD and/or concomitant diseases occurring during the post-weaning period, but without success due to the emergence of multiple antibiotic resistance. The weaning period is associated with higher antibiotic use in pig production. Use of antimicrobial agents as growth promoters or for prophylactic treatment and addition of high levels of heavy metals in the feed are still allowed in North America, but there is now growing resistance due to recrudescence of antibiotic resistance, allergic reactions to antibiotic residues, and contamination of cultivated soil. Similar regulations as those found in Europe are shortly expected in North America. Thus, there is an urgent need for alternatives to antimicrobial agents to prevent the PWD and to promote growth of pigs.

In theory, injectable vaccines, such as those used for the prevention of neonatal diarrhea and administered to sows, could be a potential solution for the control of PWD. On the other hand, injection of each piglet would not be cost-effective in production. Furthermore, these vaccines stimulate production of systemic antibodies which do not reach bacteria in the intestines. Killed vaccines or bacterins (dead bacteria) orally or parenterally administered are not efficient for the control of PWD (Husband, 1993). Thus, to protect weaned pigs, an oral live vaccine using non-pathogenic F4-positive *E. coli* must be developed.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method for enhancing animal weight gain.

More specifically, the method of the invention comprises the step of administering to an animal in need thereof an effective amount of at least one non-pathogenic F4-positive live *E. coli* strain.

BRIEF DESCRIPTION OF A PREFERRED EMBODIMENT OF THE INVENTION

Introduction

Prevention of PWD

Moon (1990) suggested the use of a live non-pathogenic *E. coli* strain for the prevention of the PWD in pigs. Although it is well accepted that a non-pathogenic F4-positive *E. coli* strain would stimulate a good humoral immunity, there are only few studies in the literature relating to trials using this kind of strain as an oral vaccine for pigs. Before Moon's recommendations, Bijlsma et al (1987) reported use of a non-enterotoxigenic F4-positive *E. coli* strain as a vaccine for sows administered before farrowing. They evaluated the transfer of the systemic antibodies from the sow to the colostrum and milk and then, to piglets during suckling. They administered 5 X 10^{11} bacteria per day during the 28 days before farrowing. No clinical signs were observed in the sows despite repetitive administrations of massive doses bacteria. They observed that this vaccination allowed production of systemic antibodies (IgG, IgM and IgA), these antibodies being transfered into the colostrum and milk, then to piglets.

Many papers reported studies related to non-enterotoxigenic F4-positive *E. coli* strain 2407, first reported by Casey and Moon (1990). This recombinant strain was created by the addition of a plasmid coding for F4 fimbriae to a wild type non-enterotoxigenic F4-negative *E. coli* strain. A similar genetically transformed strain (strain WBL1212:pMKO05) had been reported earlier (Dougan et al, 1986, Smith and Huggins, 1978, Smith and Linggood, 1971). *Vijtiuk* et al. (1995) reported that the strain 2407 would probably be a good candidate for an oral vaccine for the prevention of the PWD in pigs. This strain caused no, or very few, histopathologic changes in the intestines but conferred no, or only partial, protection against F4-positive ETEC (Francis and Willgohs, 1991, Bozic et al., 2002). Although the authors associated that lack of protection to immaturity of the immune system of piglets at weaning, stress, and/or mucosal immune system tolerance of F4 antigen, it is most probable that it is linked with the massive dose (10^{10}) of challenge ETEC bacteria used for the trials.

Bozic et al (2003) reported that levamisol, a deworming drug with immunomodulatory affects, administered before vaccination, increased the protection properties of the strain 2407 and decreased the

presence of clinical signs in pigs. Unfortunately, Bozic et al. (2003) did not test for the F4-receptor status of pigs. In fact, differences observed could be linked to the proportion of pigs with receptors, these pigs being sensitive to F4-positive ETEC infections. Surprisingly, Bozic et al (2002) reported clinical signs (diarrhea and weight loss) starting the day following the administration of the non-pathogenic strain 2407. It is possible that the strain 2407 was incorrectly characterized and possesses virulence factors or that pigs used in the trials were colonized by F4-positive ETEC from the beginning of the experiments.

Many authors have reported the use of subunit vaccines (F4 fimbriae), orally or parenterally administered, for the prevention of PWD (Snoeck et al., 2003, van der Stede et al., 2003 and 2002, Van den Broeck et al, 2002 and 1999, Bianchi et al., 1996). These intramuscularly injected vaccines are similar to those previously described for the prevention of neonatal diarrhea and administered to sows. Although rapid humoral and/or systemic immune responses were reported in these studies, only a few authors evaluated the protection properties of these vaccines. Van den Broeck et al. (1999), using a porcine PWD model, reported that an oral vaccination with F4 fimbriae eliminated the excretion of the ETEC challenge strain in the feces. Unfortunately, the clinical signs were not evaluated in this study.

Weight gain

There is no study in the scientific literature related to a wild type or recombinant non-pathogenic F4-positive *E. coli* strain inducing growth promotion in pigs.

Related commercial products

There is no veterinary or human commercial product with any relationship to a live non pathogenic F4-positive *E. coli* strain. On the other hand, there are 3 products using live non-pathogenic F4-negative *E. coli* strains:

1) Mutaflor of ARDEYPHARM (Germany; www.ardeypharm.de): Freeze dried culture of live non-pathogenic *E. coli* Nissle 1917 strain used as a probiotic in humans. Mutaflor is recommended for cases of ulcerative colitis, diarrhea, constipation, and for activation of the infant immune system. ARDEYPHARM mentions in the web site «www.ardeypharm.de: The historical development of Mutaflor therapy » that Mutaflor was tested for

food animal production, but without giving any references. Furthermore, no study has been found in the scientific literature with regard to the use of this strain in food animal production.

2) Probactrix of BioBalance Corporation (Israel; www.thebiobalancecorp.com): Liquid preparation of the non-pathogenic *E. coli* M-17 strain (ATCC 20226) mixed with volatile fractions of plant extracts. Probactrix is recommended as an exclusive competition agent for animals and humans.
    a. From the web site: "The formula was approved as a food supplement for human use by the Ministry of Health of Israel in May 1998 and was approved for use as an animal food by the Ministry of Agriculture of Israel in September 1998. The formula has been extensively tested and used on animals (poultry and piglets) as well as on thousands of human volunteers with known gastrointestinal disorders. BioBalance is not aware of any side effects occurring as a result of the use of the product."
    b. This product has been tested during the post-weaning period in pigs (www.isrvma.org/article/57_4_1.htm). They reported that Probactrix reduced the incidence of diarrhea by 6,6% and the incidence of the mortality associated with diarrhea by 6,59%. Furthermore, the authors reported higher weight gain (nearly 10%) for the animals treated with Probactrix when compared to untreated animals, but without statistics. Pigs were not challenged with an ETEC strain in this study. Diarrhea was associated with different pathogens such as *E. coli*, *Salmonella, C. perfringens,* and rotavirus.

3) Colinfant newborn of DynTec (Czech Republic; www.dyntec.cz) : Oral suspension of a freeze dried preparation of an *E. coli* O83 :K24 :H31 strain for the prevention and the treatment of nosocomial infections, intestinal infections, and intestinal bacterial populations disorders in infants.

Examples

Freeze dried pure culture of a live wild type *E. coli* strain to prevent PWD and to promote growth of pigs. This strain is non-enterotoxigenic, expresses the F4 attaching factor, and possesses no known virulence factor-associated genes (see data sheet). Particular clones of the strain, for which the F4 fimbriae expression was stable after fermentation, were selected following repeated *in vitro* passages.

Oral administration of this strain prevents PWD in pigs (annexe 1) and promotes growth of healthy pigs (annexe 2). The strain is administered via the water supply system allowing a rapid treatment to several thousand pigs at the same time, such as done for administration of soluble antibiotics.

DATA SHEET	
	Determined with standard method of serotyping using "O" antigen and at the « Statens Serum Institut 5 Artillerivej 2300 Copenhagen S Denmark »
PATHOTYPE	NÉGATIVE FOR THE FOLLOWING TOXINS • STa, STb, LT, VT1(SLTI) et VT2 (SLTII), and CNF NÉGATIVE FOR THE FOLLOWING VIRULENCE FACTORS • Eae, Pap, Paa, East-1, and Aida Confirmation by PCR and colony hybridization
MEDIUM FOR FIMBRIAE EXPRESSION	
ANTIBIOGRAM RESISTANT TO:	Ampicillin, tetracycline, spectinomycin, tiamulin, tylosine
ANTIBIOGRAM SENSIBLE TO:	Apramycin, ceftiofur, cephalothin, gentamicin, neomycin, trim/sulfamethoxazol
STORAGE MEDIUM	Freeze dried (lyophilization) medium : 5% dextran T-40; 7% saccharose, 1% monosodium glutamate
SOURCE OF THE ISOLATE	Isolated at The *Escherichia coli* Laboratory, Fac. méd. vét., Saint-Hyacinthe, 1999, from feces of a normal pig.

Annexe 1

Efficacy and innocuity studies of the strain ECL-1000 (coliPROtec) for the control of post-weaning diarrhea in pigs.

1- Problematic

Post-weaning diarrhea (PWD) is one of the most costly diseases in pig production. The PWD is usually observed during the 2 first weeks after weaning. It is generally caused by *E. coli* of serotype O149:K91 having the F4 (also named K88) attaching factor, and a combination of several toxins, such as LT, STa, and STb. For the development of the disease, pigs must have the receptors specific for the bacterial F4 fimbriae. Nearly 30 to 40% of pigs have these receptors. Mortality associated with PWD is approximately 3% but can reach 30% on a farm experiencing an epidemic episode. Furthermore, the morbidity can be very high, causing a delay in growth, thus resulting in severe economic losses for the producers. Antibiotics and high concentrations of zinc oxide are generally used to attempt to control PWD in pig production and secondary PWD associated diseases. On the other hand, *E. coli* PWD associated strains are resistant to the commonly used antibiotics in pig production. Furthermore, use of high concentrations of zinc oxide is much debated due to environmental pollution by heavy metals.

2- Objective

Evaluate the ability of a live *E. coli* F4-positive non-pathogenic strain (coliPROtec) to protect weaned pigs against an enterotoxigenic *E. coli* O149:K91 strain in a porcine PWD animal model developed in the *E. coli* Laboratory (ECL) of the Faculté de médicine vétérinaire of the Université de Montréal.

3- Experimental procedures:

3.1 Animals

Three trials were done, using 10 treated and 10 untreated pigs each, for a total of 60 pigs. Pigs with diarrhea before the challenge, colonized by F4-positive *E. coli* strain before treatment, or colonized by an enterotoxigenic *E. coli* strain before challenge were excluded of the experiment. Pigs were purchased from different commercial farms.

3.2 Porcine PWD animal model

Seventeen day-old weaned pigs were transferred in the experimental facilities of the Faculté de médecine vétérinaire of the Université de Montréal.

- Days 5 and 12 post-weaning (PW):
  - Treated animals:
    - Oral administration of a single dose of coliPROtec (non pathogenic F4-positive/enterotoxin-negative *E. coli* strain in TSB)
  - Untreated animals :
    - Oral administration of TSB
- Days 26 to 28 PW :
  - Challenge with the enterotoxigenic strain
- Day 29 PW :
  - Necropsy of animals

3.3 Evaluated parameters during experiments

Before the day of necropsy

- General health of the animals
- Diarrhea scores during the challenge days
- Weight of the animals

At the day of necropsy

- Diarrhea scores
- Consistency scores of intestinal content (Jejunum, Ileum, caecum, colon, rectum)
- Ileal colonization by the challenge strain (bacterial count)

Post-mortem

- F4-receptor status of animals

Consistency scores of feces (diarrhea)
(0): Normal
(1): Softer than normal without liquid
(2): Mainly solid with presence of liquid
(3): Mainly liquid with presence of solid particles
(4): Totally liquid

Consistency scores of intestinal contents
(0): Normal
(1): Softer than normal without liquid

(2): Mainly solid with presence of liquid
(3): Mainly liquid or totally liquid

4. Results

The results are presented for F4-positive and negative animals, then for all animals. Only animals for which it was possible to observe feces were taken into account for the diarrhea scores. Results on the weight gain are presented separately (see accompanying document).

4.1 Animals

Following exclusion of some animals with regards to criteria described previously, 28 pigs constituted the control untreated group (8 with and 20 without the F4-receptor) and 25 pigs constituted the treated group (11 with and 14 without F4-receptors).

4.2 F4-positive and F4-negative animals

4.2.1. Diarrhea scores (mean)

Animal status	Group	Day 1 post-challenge	Day 2 post-challenge	Day 3 post-challenge (Necropsy)
R-F4 positive	Control	1,63	1,75	2,14
	coliPROtec	0,18	0,36	1,09
R-F4 negative	Control	0,85	0,30	0,94
	coliPROtec	0,00	0,93	1,00

4.2.2. Consistency scores of intestinal contents (mean)

Animal status	Group	Jejunum	Ileum	Caecum	Colon	Rectum
R-F4 positive	Control	2,75	3,00	2,75	1,75	1,63
	coliPROtec	0,38	0,30	1,00	1,00	0,64
R-F4 negative	Control	0,70	1,81	0,85	0,25	0,20
	coliPROtec	0,43	0,55	0,23	0,23	0,15

4.2.3. Ileal colonization by the challenge strain (median)

Animal status	Group	CFU/g of tissue
R-F4 positive	Control	$1,1 \times 10^7$
	coliPROtec	$1,4 \times 10^5$
R-F4 negative	Control	$1,3 \times 10^3$
	coliPROtec	$3,8 \times 10^1$

4.3 All animals

4.3.1. Diarrhea scores (mean)

Group	Day 1 post-challenge	Day 2 post-challenge	Day 3 post-challenge (Necropsy)
Control	1,30	1,00	1,52
coliPROtec	0,08	0,68	1,05

4.3.2. Consistency scores of intestinal contents (mean)

Group	Jejunum	Ileum	Caecum	Colon	Rectum
Control	1,29	2,21	1,39	0,68	0,61
coliPROtec	0,38	0,76	0,58	0,42	0,25

4.3.3. Ileal colonization by the challenge strain (median)

	CFU/g de tissue
Control	$9,1 \times 10^3$
coliPROtec	$6,1 \times 10^1$

4.4. Interpretation of the results

The treatment with coliPROtec reduced by 2 Logs the colonization by the enterotoxigenic strain of the ileum of F4-receptor positive animals. Furthermore, coliPROtec nearly abolished the ileal colonization for F4-receptor negative animals.

F4-receptor positive animals treated with coliPROtec showed diarrhea and consistency intestinal content scores similar to those observed for F4-receptor negative animals.

All animals treated with coliPROtec were in good health and showed a better weight gain than untreated animals (see accompanying document for the weight gain).

We conclude that coliPROtec is safe, efficient for the control of PWD in pigs and results in a better weight gain. The administration of coliPROtec does not require any animal restraint, thus reducing animal stress. The strain coliPROtec can be easily and rapidly administrated to a large population of pigs via the water medicator system.

5.0. Conclusions

coliPROtec is efficient for the control of the PWD when 2 doses are administered at 21 and 14 days before challenge. Additional trials will be necessary for the evaluation of the administration a single oral dose or 2 oral doses close to challenge.

Annexe 2

Effect of coliPROtec on weight gain

1. Description of the product:

Live *E. coli* F4-positive non-pathogenic strain, orally administrated in weaned pigs for the control of post-weaning diarrhea (PWD) and for the promotion of the weight gain.

2. Identification of the strain

Reference number in the ECL strain collection: ECL 1000
Name of the product: coliPROtec

Note: The strain was previously designated as « JFF4 vaccine ».

3. New application of the coliPROtec

During the innocuity studies of the coliPROtec, we noticed a positive effect of the product on the weight gain of the animals. Pigs treated with only one oral dose of coliPROtec at the beginning of the post-weaning period had a daily weight gain higher by 53g when compared to untreated animals. Specifically, treated pigs were 849g heavier than untreated pigs, 2 weeks after the treatment.

Therefore, coliPROtec responds to 2 needs in pig production, control of the PWD and an additive positive effect on weight gain. Contrary to the effect on the control of PWD, the effect on weight gain has not yet been disclosed.

5. Summary of the experiments:

5.1 Animals : Five trials were done for a total of 45 treated and 45 untreated pigs. These pigs came from different commercial farms. They received standard commercial feed without antibiotics and zinc oxide.

5.2 Place : Faculté de médecine vétérinaire, Université de Montréal

5.3 Trials

Day 1 : Arrival of the 17-day-old weaned pigs.

Days 1 to 4 : Adaptation period

Day 5 : Treatment of the animals with a single dose of coliPROtec (non pathogenic F4-positive/enterotoxin-negative *E. coli* strain in TSB) and weighing of the animals. The control group was treated with TSB only.

Day 20 : Weighing of the animals and end of the experiment.

5.4 Results:

Table1 : Weight of the animals

Day	Weight (Kg)		T-test	coliPROtec vs untreated (g)
	Untreated	coliPROtec		
5	6,053	6,137	p = 0.754	+84
20	12,055	12,904	p = 0.020	+849

Table 2 : Daily weight gain

Days	Daily weight gain (g)		T-test	coliPROtec vs untreated
	Untreated	coliPROtec		
5 to 20	405	458	p = 0.007	+53

At day 5 post-weaning, before the administration of coliPROtec, the weight of the two groups was not statistically different. On the other hand, at day 20 post-weaning (15 days after the treatment), the treated animals were 849g heavier and demonstrated a daily weight gain of 53g more than untreated animals, these differences being statistically significant.

Antibiotic growth promoters generally increase the weight gain by 3,3 to 8,8% (Doyle, M.E., Food Research Institute, University of Wisconsin, 2001). The coliPROtec strain increased the daily weight gain by 11% during the 2 weeks following the single dose.

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